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13. ABSTRACT (Maximum 200 Words)

The focus of this grant was to evaluate the DNA repair pathway as a new target for therapeutic intervention and to identify inhibitors of the two pathways of double stranded break repair - Homologous Recombination and Non Homologous End Joining. Most therapeutic agents for breast cancer function by causing DNA damage, either directly (ionizing radiation) or indirectly (topoisomerase inhibitors). The problem with these agents is the generalized toxicity of the treatment. Therefore any agent that can specifically target the breast tumor can be used to sensitize the tumor alone to the DNA damaging agent.

We used virtual ligand screening using the DOCK software and 3D structure databases from Available Chemicals directory and National Cancer Institute to identify potential disruptors of the Ku 70/80 DNA binding activity. The top scoring hits from this screen were then tested for biochemical activity using an assay developed inhouse. We have identified and validated 4 novel inhibitors of the Ku complex activity. We plan to test these inhibitors as sensitizing agents to topoisomerase inhibitors in the coming months.

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Introduction:

Tumorigenesis is the result of multiple genetic changes. Although cells are subject to a multitude of environmental and chemical factors, the cell's robust repair machinery is able to repair most of the damage and, if not, at least program the cell to undergo apoptosis, thereby preventing uncontrolled proliferation of DNA damaged cells. However, mutations in these check-point genes that diminish the cell's ability to do either of these functions may lead to increased susceptibility to neoplasias. Hereditary Nonpolyposis colorectal cancer syndrome (HNPCC) is an example of such an inherited mutation causing increased susceptibility to cancer[1].

Homologous recombination is one of the important repair pathways that guards against tumorigenesis. While its prominent function is the exchange of information during meiosis, its has been shown to be a key pathway in DNA repair in bacteria and yeast[2]. In bacteria, it has been shown that Rec A is a critical component of the SOS response to ionizing radiation. In *Saccharomyces cerevesiae,* the Rad 51 and Rad 52 proteins have been identified as important players in this pathway. Rad 51 is the yeast homologue of the bacterial Rec A protein[3-5]. A mammalian homologue of Rad 51, with high homology to the bacterial Rec A protein, has also been cloned. The high degree of conservation between prokaryotes and high order eukaryotes suggests the importance of this pathway for the cell.

The other major pathway for Double Strand Break (DSB) repair is the Ku70/80-mediated Non Homologous End Joining pathway (NHEJ). DNA lesions are recognized by the Ku70/80 hetrodimeric protein which then recruits the repair complex to the lesion. Important members of the repair complex are XRCC4-DNA ligase4, which joins the broken strands of the complex and the Mre11 nuclease, which cleans the ends for ligation.

Most therapeutic agents for breast cancer function by causing DNA damage, either directly (ionizing radiation) or indirectly (topoisomerase inhibitors). The problem with these agents is the generalized toxicity of the treatment. Therefore any agent that can specifically target the breast tumor can be used to sensitize the tumor alone to the DNA damaging agent.

The focus of this grant was to evaluate the DNA repair pathway as a new target for therapeutic intervention and to identify inhibitors of the two pathways of double stranded break repair - Homologous Recombination and Non Homologous End Joining. We had proposed in the original grant application to screen for inhibitors of Rad51 multimerization thereby disrupting homologous recombination, and thus decrease the

efficacy of DNA repair. We modified our aims to include the Ku70/80 DNA binding activity and the NHEJ pathway of DNA repair[6]. We argued that deficiency in either patway of DNA damage repair will sensitize cells to DNA damaging agents and thus such tumors potentially can be treated with a lower dose of chemotherapeutic agents/radiation[7, 8].

Body

Part1: Screening for Inhibitory peptides Of Rad51 multimerization:

In Year1 we had established the initial screeing conditions for using the Reverse Yeast two hybrid system to identify inhibitory peptides of Rad51 multimerization. We had succeeded in achieving the following objectives for year1.

1. A random DNA library of complexity in the order of 10^6 encoding 15 amino-acid peptides was synthesized.
2. A system to select inhibitors of Rad51 self association in the context of a Reverse Two hybrid System was set up.

We wanted to increase the complexity of the library to above 10^7 before starting the screening process. We were able to achieve this by modifying PCR and cloning conditions. Also we were able to reduce the percentage of short/insertless clones in the library. We then transformed the library into the strains we had developed (MV103/pMAD51) as described in the Year 1 report. We had distinct problems at this stage that we were unable to overcome.

The transformation efficiency in yeast is very low. We were not able to obtain efficiency of more than 10^5. This meant that we could never screen the whole range of the possible peptides encoded by our library. The number of clones that we were able to isolate were too few in number. When the plasmids from these were rescued and reintroduced in the parent strain of yeast, there was no inhibition of Ura3 activity. So all these strains didn't survive FOA selection. In short these were determined to be false positives. We decided to shelve this part of the project due to these technical difficulties that we could not overcome.

We then focused on the additional objctives mentioned in the year 1 review of the project i.e. targeting the Ku70/80 complex for disrupting the NHEJ pathway of DNA repair. We had a lot more success in this part of the project.

Part2: Structure Based Design for KU70/80 inhibitors.

As reported in our previous report, the Ku70/80 crystal structure has recently been published[9]. For reasons outlined in our report, we wanted to make use of these data to address our overall objective i.e to design inhibitors of DNA repair as sensitizing agents to chemotherapy. For this part of the project we had accomplished the following goals at the end of Year1.

1. Critical interactions for Ku70/80 DNA binding activity were mapped.
2. A Pocket designated 157E containing amino acids critical for interactions with the DNA backbone was chosen

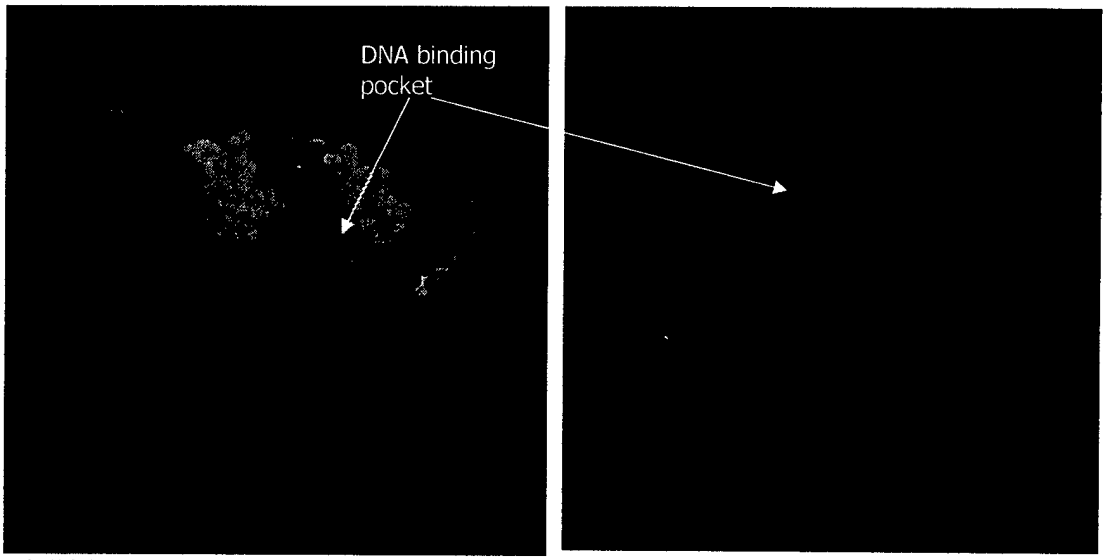

Fig 1. DNA binding pocket of Ku70/80 dimer. The structure is pared down to show the DNA binding region of the heterodimer

We used the DOCK 4.0 program to screen small molecule compound libraries for binding affinity to the DNA binding site on the Ku70/80 complex[10-12]. The compound libraries available to us were the Available Chemicals Directory (ACD) structure database which has over 300,000 compounds, and the NCI 3D database, which has around 250,000 compounds. The computational facilities for this part were kindly provided by Dr. Michael Johnson at the Center for Pharmaceutical Biotechnology, UIC. We proposed the following objectives for year 2 of the grant.

Proposed objectives for Year 2:

1. To set up a DOCK screen for small molecules targetting the DNA binding region of the Ku70/80 complex.
2. To select top hits of the screen and verify biochemical activity in the DNA binding assay for Ku70/80 complex described above.
3. To test efficacy of such inhibitors in sensitizing tumor cells to double strand breaks in DNA.

Aim 1:

To screen for potential inhibitors of the Ku70/80 DNA binding activity through Virtual Ligand screening using DOCK.

Results:

As proposed, we set up DOCK screens for the DNA binding pocket of the KU70/80 complex. The DOCK software consists of three main programs. The first step is to generate a molecular surface for the active site. This is performed using Mike Connolly's *molecular surface* (*ms*) program provided within DOCK. Next the *Sphgen* provides a negative characterization of the target site. The shape of cavities in the receptor is used to define spheres; the centers of the spheres become potential locations for ligand atoms. Finally in the *DOCK* program, sphere centers are matched to the ligand atoms, to determine possible orientations for the ligand. Typically on the order of tens of thousands of orientations are generated for each ligand molecule. Each oriented molecule is then scored for fit. There are 3 scoring schemes:

1. Shape scoring, which uses a loose approximation to the Lennard-Jones potential
2. Electrostatic scoring, which uses the program DELPHI to calculate electrostatic potential
3. Force-field scoring, which uses the AMBER potential.

As determined previously, the region used for the screen was Pocket 157E in the DNA binding region of the complex (Fig1).

DOCK requires a database of ligands from which to screen and score the binding potential to the target site. We used two sets of ligand databases for this purpose – the Available Chemicals Database (ACD) which includes about 350 000 compounds and the NCI3D database which includes about 220 000 molecules that have been screened for anticancer activity in the NCI dvelopmental therapeutics program.

Among the top scorers were a variety of nucleoside analogs (UTP, dATP), which served as internal positive controls and provided validation for the scoring functions, as well as the target site on the Ku complex. The top 500 chemical scores from each of these screens were then clustered according to structural diversity using the Insight II package. Forty compounds (20 from ACD and 20 from NCI3D) were selected so as to represent all different structural classes to test for biochemcal activity.

Aim 2:

To develop a biochemical assay for testing/screening potential inhibitors of Ku70/80 activity.

To test the high scoring hits from the DOCK screening for inhibition of Ku70/80 activity we needed to develop a quick assay. We used streptavidin beads to immobilize 5' biotinylated double stranded DNA that was used to pull down Ku70/80 complexes from total cell extracts from MCF7 cells. The pulled-down proteins were separated on a 7.5% polyacrylamide gel. The Ku70 or Ku80 can then be detected by western blotting.

However if any test compound inhibits this activity, the complex is not pulled down (Fig 2). We then showed that this assay is semi-quantitative and can be used to quickly screen a large number of compounds for biochemical activity (Fig 3).

It should be noted that the assay conditions require a large amount of protein and DNA. This is beacause of the low efficiency of Ku70/80 pull-down by the double stranded DNA. The concentration of compounds to be tested should be appropriately high.

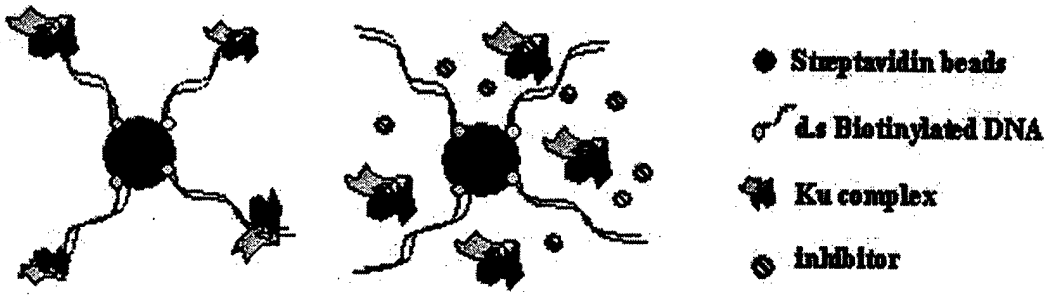

Fig2: Principle of the Ku70/80 DNA binding activity assay

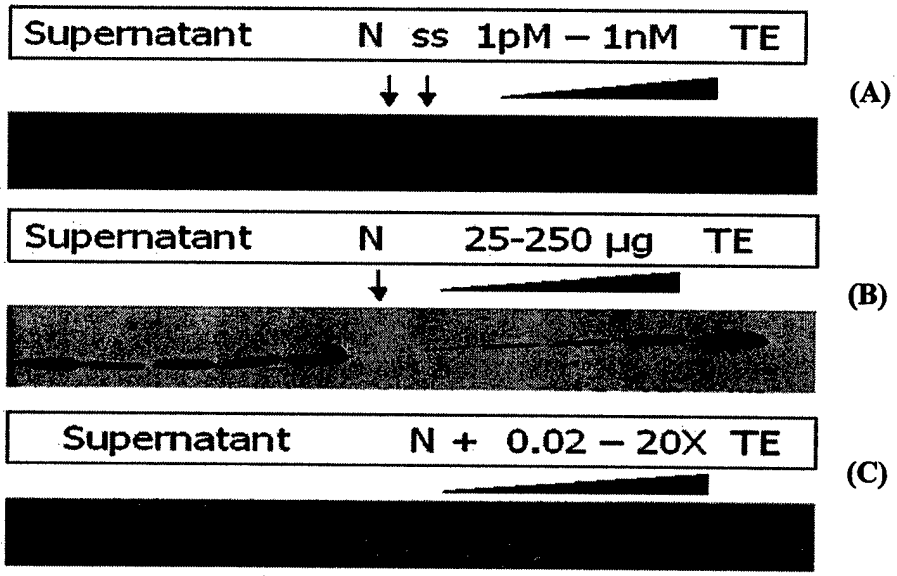

Fig 3. Ku70/80 DNA binding assay. (A) Increase in amount of double stranded DNA increases Ku complex pulled down, (B) Increase in Total protein used increases amt if Ku complex pulled down, (C) Amount of Ku complex pulled down can be competed out by free double stranded DNA in solution (N – Negative control, TE – Total Extract, ss - single stranded DNA)

Aim3: To test high scoring hits from the DOCK screen for biochemical activity

We used the assay described above to test the putative inhibitors of Ku70/80 DNA binding activity. We used 100μg of total protein extract from MCF7 cells and 10 pm of biotinylated DNA for the assay. The compunds to be tested were dissolved in DMSO and tested at three different concentrations (75μM, 150μM and 225μM). Four compounds out of 40 tested had biochemical activity. As noted above, the concentrations at which the compounds were tested was high. This does not reflect the concentrations at which these compounds need to be tested in cell line or other biological systems. It merely reflects the sensitivity of the assay. One ACD compound (Berryllon II) and 3 NCI compounds (NCI 37052, NCI37203 & NCI 634748) inhibited Ku activity (Fig 4, Fig 5).

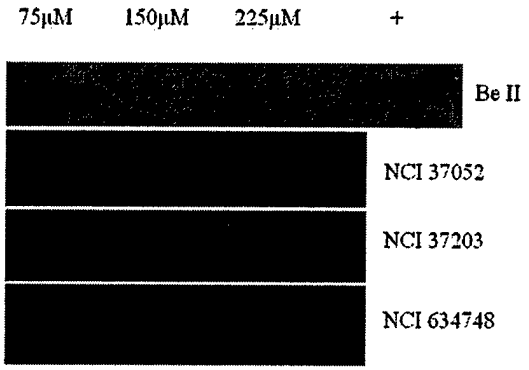

Fig 4. Compounds having biochemical activity. 100μg of total protein extract and 10 pm of biotinylated DNA were used for the assay. Compounds were dissolved in DMSO and tested at atleast 3 different concentrations (75μM, 150μM and 225μM).

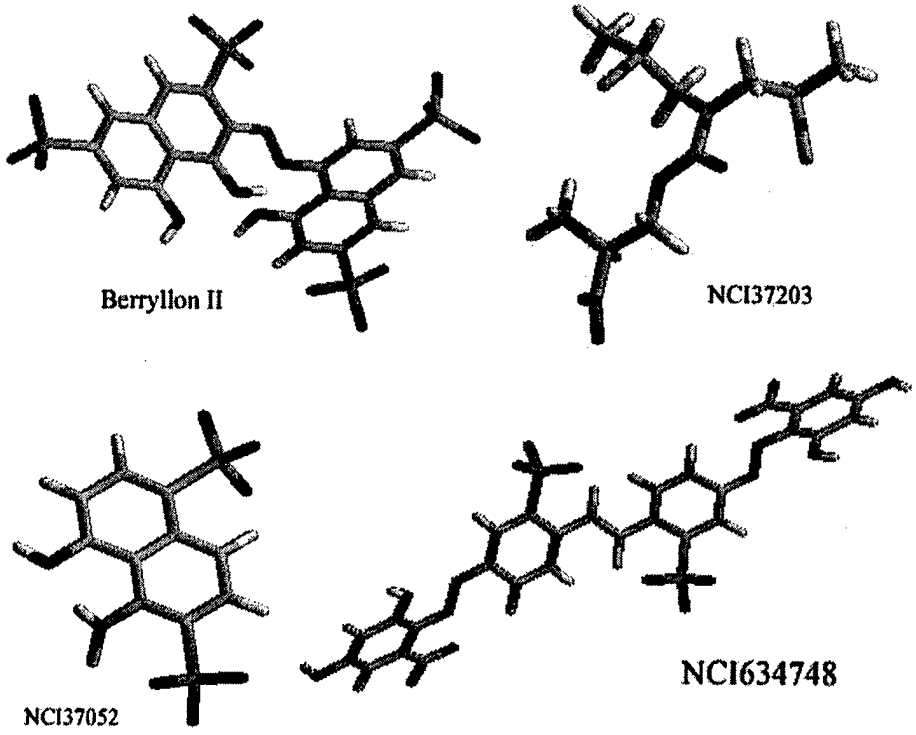

Fig 5. Structures of biochemically active compounds

Aim 4: To test whether the inhibitors of Ku70/80 activity disrupt protein protein interactions.

We needed to test the specificty of the inhibition of the Ku activity by these compounds. Since the inhibitors were all charged molecules, there was a possibility that this was a non-specific effect mediated by the general disruption of any protein-protein ineteractions. To test this, we carried out immunoprecipitation reactions using antibodies against Ku80 and then probed for Ku70 as well as Ku80. There was no difference between the control reactions and the immuoprecipitations carried out in the presence of 300μM of test compounds. This proved that the inhibition was not due to the disruption of antibody-protein or other protein-protein interactions and also eliminated protein folding as a confounding factor (Fig 6).

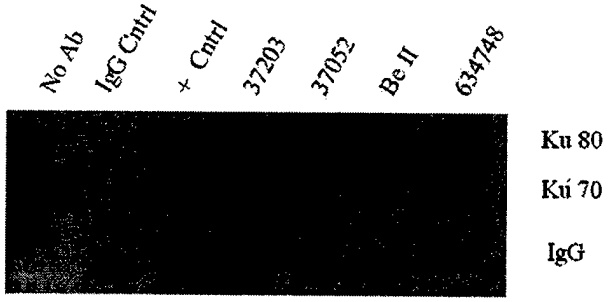

Fig 6. Inhibitors of Ku activity do not destroy protein-protein and protein-antibody interactions. Immuno precipitation reactions were carried out in the presence of 300μM of test compounds (Berryllon II, Nci 37203, 37052 & 634748) or diluent (DMSO) using mouse monoclonal antibody against Ku80. The precipitated proteins were then separated using PAGE. Westerm blotting was caried out using anti Ku70 and anti Ku80 antiboides. IgG heavy chain served as a loading control.

Objectives for Year 3:

1. Test specificity of the inhibition of Ku activity, by assaying for disruption of DNA binding activity for other nonspecific DNA binding proteins such as histones and DNA polymerase
2. To test sensitization of tumor cell lines to DNA damaging agents (topoisomerase inhibitors, γ radiation)

Key Research Accomplishments:

1. DOCK screens were carried out to identify potential inhibitors of the Ku70/80 DNA binding activity.
2. An assay for testing the inhibitors of the Ku activity was established.
3. 40 compounds selected from the top hits from the DOCK screen were tested for biochemical activity in the assay developed inhouse.
4. Four novel inhibitors of the Ku70/80 activity were identified.

Reportable Outcomes:

Poster: S. Kamalakaran, W. TaoFu, M. Johnson, & WT Beck. Identification of inhibitors of Ku70/80 activity by Virtual Ligand Screening, AAPS Pharm Sci., Vol.4, No.4, M1182, 2002.
Invited Presentation: S. Kamalakaran, Proteins involved in DNA Repair as targets for Breast Cancer Chemotherapy, Am. Assoc. Pharm. Sci. Meeting, Toronto, Canada (2002).

Conclusions:

Due to inherent technical issues with the reverse yeast two hybrid system, we were not able to successfully screen for inhibitory peptides against Rad51 self association.
However we succeeded in achieving our objectives for the second part of the project – identifying inhibitors of Ku70/80 DNA binding activity through Virtual ligand screening. We have identified and validated fourq novel inhibitors of the Ku complex activity. We plan to test these inhibitors as sensitizing agents to topoisomerase inhibitors in the coming months.

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